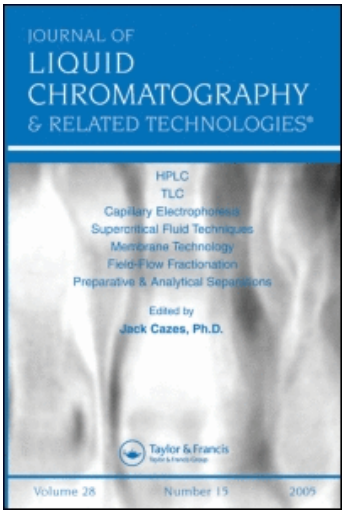

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Purification of Phosphoenolpyruvate Carboxylase and Characterization of Changes in Oligomerization Using HPLC

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PURIFICATION OF PHOSPHOENOLPYRUVATE CARBOXYLASE AND CHARACTERIZATION OF CHANGES IN OLIGOMERIZATION USING HPLC

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ABSTRACT

Maize leaf phosphoenolpyruvate carboxylase and its subunits were chromatographed and extensively purified from crude preparations of the enzyme on a Bio-Sil TSK 400 column. The enzyme (tetramer) showed no adsorption to the column or loss of enzymatic activity. The tetramer complex was dissociated into its dimers and monomers when treated with the thiol oxidant diamide at pH 6.8 and 8.4. The effects of diamide were approximately twice as fast at pH 8.4 than at pH 6.8. Under either condition a certain amount of dimer still remained and could only be further dissociated by heating.

INTRODUCTION

Phosphoenolpyruvate carboxylase (PEPC) is widely distributed throughout higher plant species. In both C_4 and CAM plants, PEPC is an essential enzyme for photosynthesis since it is the first committed step for CO_2 fixation.

Abbreviations: CAM = plants which have Crassulacean acid metabolism; C_4 plants = plants which have the C_4 dicarboxylic acid pathway of photosynthesis; MW = molecular weight; DTT = dithiothreitol; MOPS-KOH = 3-(N-morpholino)-propanesulfonic acid buffer with pH adjusted with potassium hydroxide; SDS = sodium dodecylsulfate; tricine = N-tris-(hydroxymethyl) methyl glycine; Tris = tris-(hydroxymethyl)-aminomethane.

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Evidence from several sources show that PEPC is oligomeric, with the tetramer being approximately 400 kD (1). Where examined using SDS gel electrophoresis, the monomer from maize has a MW of about 100 kD. It is uncertain whether the monomer, dimer and tetramer are all active and, if so, whether there are differences in the kinetic properties of the different molecular forms. Enzymes that do exhibit changes in kinetic properties through dissociation/association are often cold labile, dissociated by dilution and have narrow ranges of pH stability (2, 3). It has been shown that PEPC from maize is cold labile (4, 5) and more stable at lower pH (6). In a recent study by Karabourniotis et al. (7), the activity of PEPC was higher when extracted from C_4 plants in the light than in the dark. The mechanism of this effect is not known, nor is it known if the enzyme undergoes changes in association/dissociation or conformation.

In our studies on PEPC from maize we have been primarily interested in characterizing the enzyme activity as it may relate to changes in association/dissociation and photoperiod. It is necessary for studies of this type that rapid isolation, purification and assay methods be used.

We describe here a fast method of purification of PEPC from maize and examine its molecular forms under varying conditions of thiol group oxidation. This system uses high performance size exclusion chromatography (HPSEC) of ammonium sulfate precipitates of the enzyme. A medium was developed for elution of the protein in an active form by using a combination of glycerol and acetonitrile which may have general application for studying proteins using HPLC. In addition, we show physical differences between the sub-units with respect to hydrophobicity and charge at different pH values due to interactions of the sub-units with the TSK column's matrix.

EXPERIMENTAL

Plant Material

Maize plants were grown as previously described (8). Young leaf tissue was used for protein isolation when 2-3 weeks old.

Protein Extraction and Partial Purification

Extraction of PEPC from maize leaves was according to the method used by Uedan and Sugiyama (4), with minor modifications. The grinding medium, and subsequent media contained 25 mM MOPS-KOH (pH 6.8) in place of Tris-HCl. After the final 70% ammonium sulfate precipitation, a portion of the pellet was desalted on a Sephadex G-25 column equilibrated with 10 mM MOPS-KOH (pH 6.8) and 5 mM DTT. The Sephadex G-25 eluate was applied to a DEAE-cellulose column equilibrated with the above buffer. The column was first washed with buffered 80 mM KCl, and the PEPC fractions were collected using 120 mM KCl.

The fractions were collected and precipitated in 70% ammonium sulphate. At this stage, the PEPC is still not free from other proteins. These proteins were eliminated by collecting the 400 kD fractions from a Bio-Rad Bio-Sil TSK 400 HPLC size exclusion column, and purified PEPC stored as 70% ammonium sulfate precipitate. Final purity of the fractions was tested by gel electrophoresis.

Enzyme assay

PEP carboxylase activity was determined spectrophotometrically at 27°C by monitoring NADH oxidation at 340 nm and coupling the PEPC reaction with malic dehydrogenase. The standard 3 ml assay medium contained 25 mM Tricine (pH 8.3), 5 mM $MgCl_2$, 0.2 mM NADH, 10 mM $NaHCO_3$, 2 units malic dehydrogenase, 4 mM PEP and PEPC (10 ug of protein). Determination of protein was performed using the Bradford method (9) with BSA as standards.

HPLC Analysis

A Bio-Rad Bio-Sil 400 TSK size exclusion column (300 x 7.5 mm) and TSK guard column were used for the HPLC analysis and protein purification, using a thermostatically controlled water jacket at 21°C. For purification, the PEPC preparation was recycled through the column a total of three times to improve separation of the individual protein.

The Bio-Sil TSK-type column was calibrated by chromatography protein standards (Sigma) and by plotting the log MW of each standard against its retention time. Thyroglobin (MW 660,000), ferritin (MW 450,000), β-amylase (MW 220,000), alcohol dehydrogenase (MW 140,000), hexokinase (MW 104,000) and BSA (MW 132,000 for dimer and 66,000 for monomer) were used. The column was equilibrated with the preferred mobile phase of 10 mM sodium phosphate pH 6.8. This mobile phase was used throughout these investigations with additions noted in the appropriate figure legends. The PEPC protein and protein standards were chromatographed at 21°C using the isocratic 10 mM phosphate mobile phase (pH 6.8), with the indicated modifications, at a flow rate of 36 ml/h.

The column was attached to a Shimadzu LC-4A chromatograph and Beckman model 160 uv detector using a zinc lamp and a 214 nm filter. Chromatograms were recorded using a Linear strip chart recorder at 1 cm/min.

RESULTS

The protein standards and a typical calibration curve are shown in Figure 1. Since detection limits may become a problem when working with small quantities of crude extracts of PEPC, routine analysis at 214 nm was used. Protein calculations were based on the assumption that at this

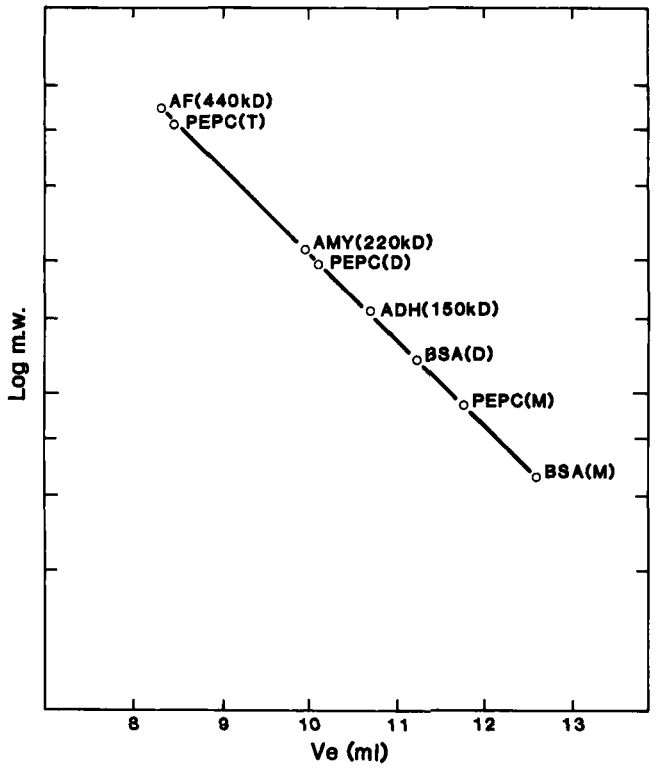

Figure 1. Calibration plot of protein MW using the TSK 400 column. Log of MW is plotted versus the elution volume (Ve). Mobile phase was 10 mM sodium phosphate buffer (pH 6.8), flow rate 0.6 ml/min. Protein standards were made up in mobile phase (1 mg/ml). AF, apoferritin; AMY, β-amylase; ADH, alcoholdehydrogenase; BSA, bovine serum albumin; PEPC, PEPcarboxylase; T, tetramer; D, dimer; M, monomer. Injection volumes were 50 ul.

wavelength, absorptivities of all species were similar (10). Thus, absorptivity changes in the different molecular forms of the enzyme at 214 nm were used to assess their relative levels under varying conditions.

It became very apparent to us that, with varying ionic strength of the mobile phase, the tetrameric, dimeric and monomeric forms of PEPC did not elute according to their respective molecular weights (when a standard calibration was used at a given ionic strength of $(NH_4)_2SO_4$). This strongly suggested that there were non-ideal size exclusion factors (n-SEC) affecting the chromatography of the PEPC complex. Pfannkock (11) has shown that these n-SEC factors are due to the packing of the column and its interactions with proteins.

Effects of ionic strength on K_T at a constant pH

Factors causing the non-ideal size exclusion (n-SEC) of maize PEPC and other proteins on HPSEC have been studied. Non-ideal size exclusion may result when mobile phases of low ionic strength cause electrostatic interactions to occur between the charged SEC packing materials and proteins (11). Mobile phases of higher ionic strengths can induce a solvophobic effect between the mobile phase and some hydrophobic proteins. These latter conditions cause the protein to adsorb to the surface of the SEC support.

The effect of increasing the ionic strength of the mobile phase at pH 6.8 upon elution of several proteins, including the dimer of PEPC, can be seen in Figure 2. This particular data was obtained using 10 mM MOPS and the indicated concentrations of $(NH_4)_2SO_4$. The changes in K_T values are plotted as a function of ionic strength where $K_T = K_s + K_i$. K_T is total capacity factor (retention factor), K_s is size exclusion factor and K_i is a capacity factor due to surface interaction (hydrophobicity, charge and bioaffinity). The values were calculated by using the following expression:

$$K_T = \frac{V_e - V_o}{V_i}$$

The term V_e, V_o and V_i represent solute elution volume, interstitial volume of the column and the pore volume, respectively. The V_o was determined with blue dextran or whole calf thymus DNA. Azide or ADP were used to determine V_i (11). The results in Figure 2A show that K_T may be experimentally affected by changes in ionic strength at a constant pH. These effects can be so pronounced as to overcome size exclusion. At low $(NH_4)_2SO_4$ (10 mM) amylase (220 kD), alcohol dehydrogenase (150 kD) and the BSA dimer (132 kD) elute at the same apparent K_T. This is also seen at 100 mM $(NH_4)_2SO_4$ with

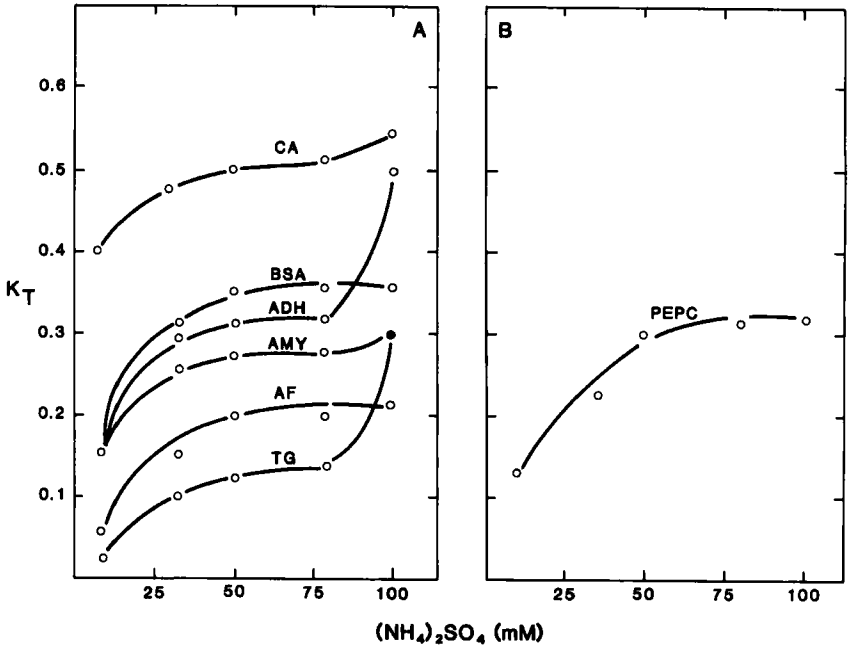

Figure 2. The effect of increasing the ionic strength of the mobile phase at pH 6.8 upon retention values (K_T) of several proteins (A) and the PEPC tetramer (B). CA, carbonic anhydrase, TG, thyroglobin. Other abbreviations are the same as indicated in the legend of Figure 1.

apoferritin (440 kD) and amylase (220 kD), where both elute before thyroglobin (660 kD). Also at 100 mM $(NH_4)_2SO_4$, BSA (132 kD) elutes prior to alcohol dehydrogenase (150 kD). Figure 2B indicates the elution of PEPC tetramer is also affected by changes in ionic strength. Between 10 mM and 50 mM $(NH_4)_2SO_4$ the tetramer's K_T value increases from 0.13 to 0.30 (Fig. 2B). If this was taken as the apparent K_T value when compared to Fig. 2A, it would reflect a change in apparent MW from 660 kD to 66 kD. In addition with increasing $(NH_4)_2SO_4$, the PEPC dimer became indistinguishable from the tetramer and the monomer's apparent K_T increased, giving unacceptable values.

Effects of varying pH on K_T at constant ionic strength

Further manipulation of the apparent PEPC K_T values is possible by changing the pH of the mobile phase at a constant ionic strength. This is

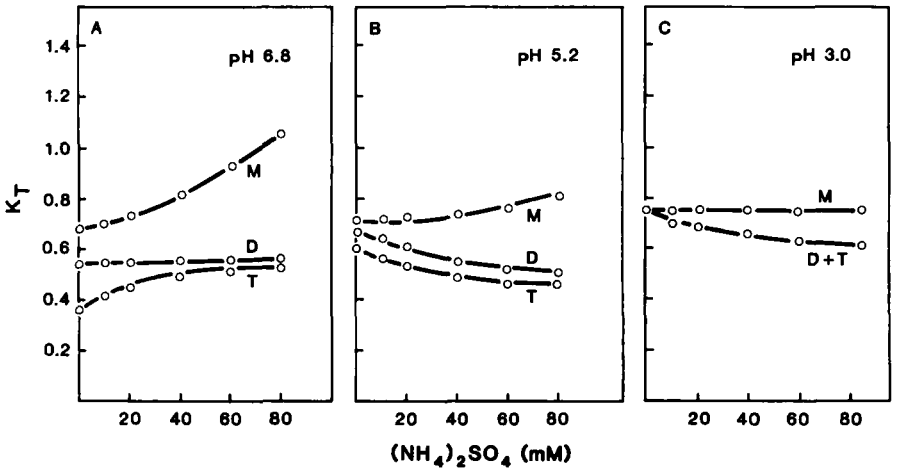

Figure 3. The effect of changing mobile phase ionic strength on the elution of the PEPC tetramer (T), dimer (D) and monomer (M) forms at pH 6.8 (A), pH 5.2 (B) and pH 3.0 (C).

thought to reflect the electrostatic charges of the proteins according to their isoelectric point (pI) values (12). Figure 3 shows the effects of varying concentrations of $(NH_4)_2SO_4$ on maize PEPC elution times at three pHs. These three pH values were chosen because the P_I of PEPC from maize is 5.2 (Ku, unpublished results). Therefore, at pH 6.8 the protein will have a net negative charge, at pH 5.2 it should be electrically neutral, and at pH 3.0, carry a net positive charge. At a low ionic strength and pH 6.8, the K_T values of the tetramer reflect maximum exclusion due to the net increase in negative charges on the protein and repulsion of the negatively charged column matrix. At higher ionic strength and pH 6.8, the monomer form was seen to elute at a range beyond the exclusion volume of the column. This indicates strong sorption of the sub-unit. At pH 5.2 and varying $(NH_4)_2SO_4$, little change was noticed between the K_T values of the tetramer and dimer. Both forms were parallel in decreasing K_T values with increasing ionic strength. This indicates some repulsive effect. The monomer form still showed retention by the column as ionic strength increased. At the lowest pH used (pH 3.0), the monomer remained at a constant kD during changes in ionic strength; while the tetrameric and dimeric forms were not distinct and showed

some exclusion. These results parallel those of Kopaceiwicz and Regnier (12) using ovalbumin and whale myoglobin.

While n-SEC is often looked upon as a drawback, it does allow for certain insights into the nature and separation of the PEPC complex. While it is not possible to say from these results what the pI value of the 100 kD sub-unit is, these experiments suggest that it is near pH 3.0, or more acidic than the tetramer and dimer. It may also be suggested that at pH 6.8 and above, the small sub-unit may go from a fibrous to a globular conformation, thus decreasing its Stokes radius or axle ratio. This, in combination with some sorption mechanism, may explain the large increase in kD. Whatever the case, the 100 kD sub-unit must be very hydrophobic with a dependence on pH and thus, its elution is very pH sensitive. From Fig. 3 it can be seen that acceptable separation can be obtained between the different conformational states of PEPC at ionic strengths of $(NH_4)_2SO_4$ of less than 20 mM. Investigations showed, however, that the recovery of the protein from the column was relatively poor, ranging from 50-60%.

Effects of Solvents on Protein Resolution and Recovery

The addition of non-ionic solvents to the mobile phase can enhance the resolution of certain protein separations on TSK-type columns. It also often increases the recovery of the protein from the column. Of interest to us was the work reported by Rivier (13) and more recently, Swergold and Rubin (14). In both of these studies, various concentrations of acetonitrile in aqueous mobile phases of low ionic strength were used. This was shown to overcome hydrophobic interactions between the proteins, as well as protein/column interactions.

Using a mobile phase of 10 mM phosphate buffer (pH 6.8) with different protein standards, there was a pronounced loss of linearity at MW larger than 200 kD and lower than 100 kD (data not shown). The addition of 15% acetonitrile to the mobile phase resulted in an extension of the linear portion of the TSK 400 column from 30 kD (carbonic anhydrase) to 660 kD (thyroglobin). Higher concentrations of the solvent (25%) resulted in a loss of resolution between the protein peaks. This is in contrast to the results of Swergold and Rubin (14), who showed optimum acetonitrile concentrations to be 35-45% in 0.1% trifluoroacetic acid. In addition, it was found that even at 15% acetonitrile there was a 30-40% inhibition of PEP carboxylase activity after 60 minutes. Fortunately, glycerol was found to protect against acetonitrile inactivation of the enzyme. The addition of 2.5% glycerol to the mobile phase resulted in no inhibition after 120 minutes and had no effect on protein separation. Subsequent experiments have shown that the

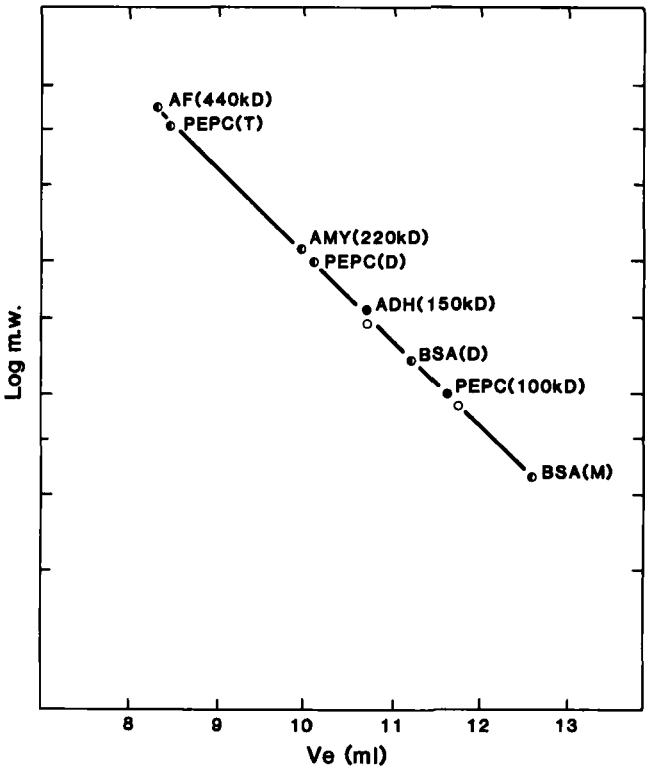

Figure 4. Calibration plot of TSK 400 column using acetonitrile (15%) and glycerol (2.5%) in phosphate mobile phase (10 mM), pH 6.8. Log of MW is plotted versus the elution volume (V_e). Protein standards are the same as in Figure 1. O,Phosphate buffer; ●,phosphate buffer and 50 mM $(NH_4)_2SO_4$.

same advantages of the acetonitrile/glycerol mobile phase system can be realized if these proportions are added to the protein sample and then injected into the column using only the 10 mM phosphate buffer (pH 6.8) for the mobile phase (Figure 4). This same figure also shows that the combination of glycerol/acetonitrile proves effective against ionic strengths up to 50 mM $(NH_4)_2SO_4$. The addition of glycerol without 15% acetonitrile to either the phosphate mobile phase or to the protein suspension resulted in loss of resolution. This latter point is important in the case of PEPC chromatography, since the addition of glycerol to the protein isolation and

suspension media has been shown to protect activity as well as to eliminate the need for DTT, therefore making it possible to observe light dependent effects (15). The role of glycerol is unclear in this respect. Without acetonitrile present, the loss of resolution may be explained as a result of increased microviscosity effects and hydrophobic interactions between the various proteins because of the ordering effect glycerol has on water (16, 17). Acetonitrile may overcome both of these factors to the extent that they do not interfere with the protein chromatography. Further experiments are needed to see if glycerol has a similar effect with HPSEC of other complex hydrophobic enzymes.

PEPC Conformational Changes and Enzyme Activity

Figure 5 is a series of chromatograms of PEPC under various states of reduction by DTT. The first trace (A) is of PEPC in the fully reduced state (5 mM DTT at pH 6.8). The sample was then treated with 10 mM of the thiol oxidant diamide (18) for 60 minutes. This resulted in the decomposition of the 400 kD form of the enzyme to the dimeric and monomeric forms (trace B). After 2 hours of this treatment, more monomer was present and a substantial decrease in dimer was seen (trace C). This treatment, however, produced no further increase in the monomer even after 4 hours (data not shown). Increases in the monomer could be seen if the sample was heated at 60°C for 10 minutes (trace D). It has been reported (6) that crude extracts of PEPC from maize leaves show considerably better stability at pH 6.8 than at the enzyme's pH optimum for catalysis (pH 8.4). A similar experiment was performed, as shown in Figure 5, but at a preincubation pH of 8.5 with the same concentrations of the enzyme (Fig. 6). It can be seen that the tetrameric form has completely disappeared at the end of 60 minutes, as well as approximately 60% of the 200 kD form (trace C). The monomer form shows a corresponding increase. Again, however, these ratios did not appear to change significantly after an additional hour of treatment with diamide. It was only with further denaturation by heating that an additional decrease in the 200 kD peak was observed (trace D). These data (Figures 5 and 6) suggest that the lower stability of the dimer of PEPC at the higher pH reflects changes that allow more rapid oxidation of the PEPC cysteine as well as possibly affecting the histidine groups (18-21). This type of instability has recently been shown to exist with the pH induced unfolding of the constant fragment of the immunoglobulin light-chain due to reduction of disulfide bonds chain observed using circular dichorism (22).

DISCUSSION

The use of size exclusion HPLC has been shown to be an excellent means of examining the state of association/dissociation of PEPC. Extraneous

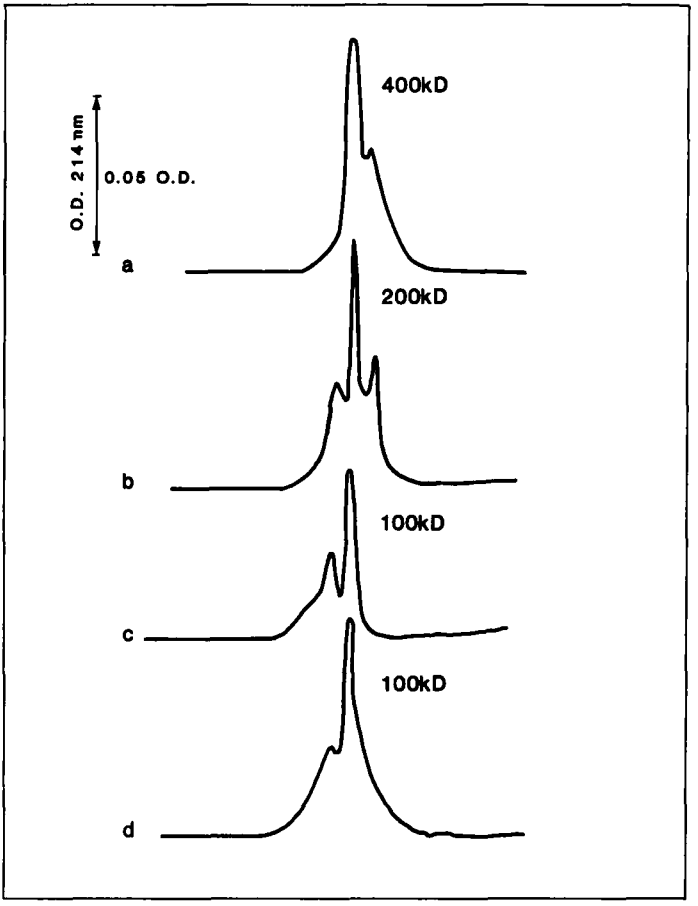

Figure 5. Oxidation of PEPC sulfhydryl groups by 10 mM diamide at pH 6.8. Chromatograms were run after preinubation (A) 60 min with 5 mM DTT, (B) 60 min with 10 mM diamide, (C) 120 min with 10 mM diamide, and (D) 120 min with 10 mM diamide followed by heating at 60°C for 10 min. PEPC protein in 70% $(NH_4)_2SO_4$ precipitate was resuspended in the mobile phase buffer plus 2.5% glycerol and 15% acetonitrile, and 20-25 ug protein was injected to the column after various treatments.

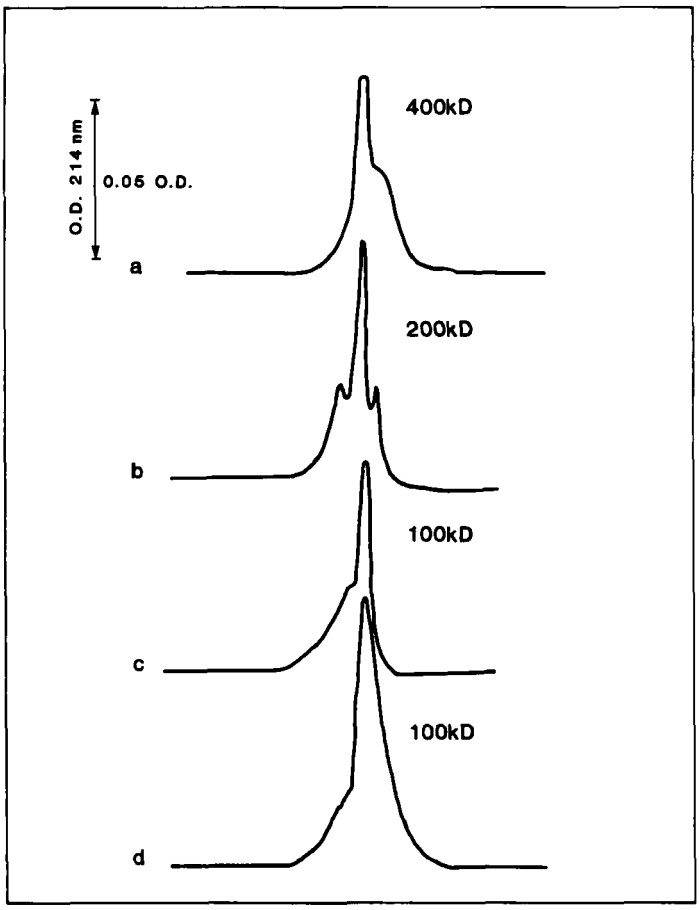

Figure 6. Oxidation of PEPC sulfhydryl groups by 10 mM diamide at pH 8.5. Chromatograms A-D are under the same conditions as shown in figure 5.

proteins can be separated and the PEPC itself recycled to give high protein purity (90-95% as verified by SDS gel electrophoresis) in very short periods of time (70-80 minutes). These fractions show little loss in activity, primarily because of rapid chromatography.

A further, and often neglected, advantage to using TSK-type columns is that under certain circumstances (low and high ionic strengths and/or varying pH) proteins separate differently. This is due to charge and hydrophobic

interaction with the column's matrix which ultimately affect the protein elution times. Although this can lead to erroneous interpretations of size, careful exploitation of these phenomena can also show qualitative differences between different sub-units of a complex enzyme/protein. In this same context, TSK-type columns have also been used to detect differences in conformational changes caused by elongation (23). This latter aspect was not a concern to us, since under the proper conditions of ionic strength and non-aqueous solvents the PEPC elution profiles did not deviate significantly from our standard protein curve or from reported molecular weight values obtained by gel electrophoresis.

We have shown that HPSEC is very useful for purifying PEPC and its sub-units for additional analysis. Also, unlike other forms of separation involving gels, it gives much better resolution. In addition, HPSEC, as opposed to reverse-phase HPLC, avoids use of strong denaturing organic solvents.

These preliminary results show the potential to use HPLC to identify the effects of several in vivo factors which may control the state of oligmerization of PEPC. It also allows for important investigations concerning different molecular forms and enzyme activity. Size exclusion HPLC has been used to study the PEPC 100 kD sub-unit of PEPC from sorghum in conjunction with immunoprecipitations (24). However, we believe our study is the first to apply this type of chromatography to a plant enzyme complex and to examine factors affecting its sub-units and their degrees of self polymerization.

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